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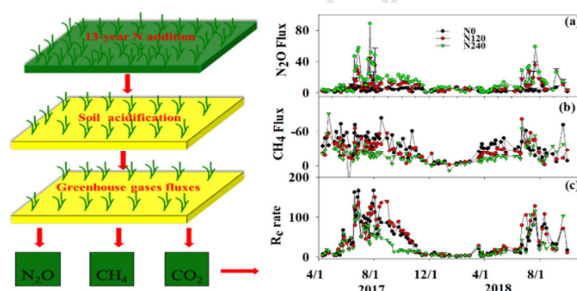
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Graphical abstract

An *in situ* experiment was conducted to investigate the effect of long-term (13-years) increased N addition on N_2O and CH_4 fluxes and R_e from a temperate grassland in Inner Mongolia, northeast China, from April 2017 to October 2018. Soil pH values in the 0-5 cm layer receiving 120 (N_{120}) and 240 (N_{240}) kg N ha^{-1} decreased from 7.12 to 4.37 and 4.18, respectively, after 13 years of N inputs. Soil CH_4 uptake was significantly reduced, but N_2O emission was enhanced significantly by N addition. However, N addition had no impact on R_e . Structural Equation Modeling indicated that soil NH_4^+ -N content was the dominant control of N_2O emissions, but with less effect of the decreasing pH. In contrast, CH_4 uptake was generally controlled by soil pH and NO_3^- -N content, and R_e by forb biomass. The measured changes in N_2O and CH_4 fluxes and R_e from temperate grassland will have a profoundly impact on climate change.



Impact of 13-years of nitrogen addition on nitrous oxide and methane fluxes and ecosystem respiration in a temperate grassland

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Abstract

Nitrogen (N) fertilizer application and atmospheric N deposition will profoundly affect greenhouse gas (GHGs) emissions, especially nitrous oxide (N₂O) and methane (CH₄) fluxes and ecosystem respiration (R_e, i.e. CO₂ emissions). However, the impacts of long-term N inputs and the often associated N-induced soil acidification on GHG fluxes in arid and semi-arid ecosystems, especially temperate grasslands, are still uncertain. An *in situ* experiment was conducted to investigate the effect of long-term (13-years) N addition on N₂O and CH₄ fluxes and R_e from a temperate grassland in Inner Mongolia, northeast China, from April 2017 to October 2018. Soil pH values in the 0-5 cm layer receiving 120 (N₁₂₀) and 240 (N₂₄₀) kg N ha⁻¹ decreased from 7.12 to 4.37 and 4.18, respectively, after 13 years of N inputs. Soil CH₄ uptake was significantly reduced, but N₂O emission was enhanced significantly by N addition. However, N addition had no impact on R_e. Structural Equation Modeling indicated that soil NH₄⁺-N content was the dominant control of N₂O emissions, but with less effect of the decreasing pH. In contrast, CH₄ uptake was generally controlled by soil pH and NO₃⁻-N content, and R_e by forb biomass. The measured changes in N₂O and CH₄ fluxes and R_e from temperate grassland will have a profoundly impact on climate change.

Key words: grassland ecosystem; long-term N addition; soil acidification; greenhouse gas emissions; influencing factors

Main finding

Long-term increased N addition significantly reduced CH₄ uptake and enhanced N₂O emissions, but had no significant impact on ecosystem respiration in temperate grassland.

Introduction

Atmospheric nitrogen (N) deposition has increased by more than 5.0 kg N ha⁻¹ over the whole global land area from 2000 to 2010 (Penuelas et al., 2013), and is expected to continue to increase until 2050 (Galloway et al., 2008; Liu et al., 2013; Xu et al., 2018). This is significantly affecting greenhouse gas emissions - nitrous oxide (N₂O), methane (CH₄) and carbon dioxide (CO₂) fluxes - in terrestrial ecosystems (Bai et al., 2010; Liu et al., 2013), which will profoundly influence climate change (Guo et al., 2010, Yang et al., 2012). Nitrogen inputs can also cause ecosystem degradation through a suite of effects that include soil acidification and eutrophication (Yang et al., 2012), with a decline or even disappearance of some plant species (Clark and Tilman, 2008; Song et al., 2012; Lu et al., 2015).

Grasslands cover approximately 40% of the global ice-free land area, account for 12% of the global carbon (C) pool, and are important sinks for GHGs (Gomez-Casanovas et al., 2016; Dijkstra et al., 2013). Any reduction in this sink is critical for climate change (Chen et al., 2013). Soil acidification is of great concern because of its impact on greenhouse gas fluxes in terrestrial ecosystems (Guo et al., 2010, Yang et al., 2012). Global soil pH values declined by 0.26 units on average as a result of N deposition during 1900 to 2014 according to a review (Tian and Niu, 2015), which has been found to shift grassland soil into the Al³⁺ and Mn²⁺ buffering stage (Tian and Niu, 2015). In temperate grassland in northeast China the decline has been especially large, 0.63 pH units since 1980 (Yang et al., 2012), which will have profoundly impacted GHGs sinks.

Soil pH has been shown to be the chief factor of global N₂O emission by selecting for specific microbial community structure (Cuhel and Simek 2011; Morales et al., 2015; Wang et al., 2018). The rate of ammonia oxidation, the main N₂O production pathway in aerobic soil, has been shown to decrease rapidly with reduced pH in culture and field experiments (O'Neill and Wilkinson, 1977; Zhang et al., 2016). N inputs increase soil N availability and simultaneously decrease soil pH, which seem to have conflict influence on N₂O production. N addition and acid deposition were shown to have opposite effects on both the abundance and activities of nitrifiers of different groups (Ying et al., 2017). In N enriched soils, ammonia oxidizing bacteria (AOB) rather than ammonia oxidizing archaea (AOA) are primarily responsible for ammonia oxidation (Di et al. 2009; Zhou et al., 2015). In contrast, AOA are responsible for ammonia oxidation in acidic grassland (Li et al., 2018), AOB are better able to utilize ammonia, and produced more N₂O through nitrification (Prosser and Nicol, 2012). In addition, denitrification rates are reduced at low pH, but N₂O production is enhanced, because the synthesis of N₂O-reductase is inhibited (Bergaust et al., 2010). Overall, soil acidification and soil mineral N content, especially NH₄⁺-N, are critically important for determining soil microbial community shifts and N₂O production in aerobic soil (Li et al., 2018), but it is still unclear whether N₂O emission is mainly regulated by soil substrates (i.e. N) or low pH in long-term N-induced acidified soil.

Some studies have indicated that long-term N addition inhibits CH₄ uptake in aerobic soils, or even changed CH₄ fluxes from sink to source in a forest soil (Liu and Greaver, 2009; Aronson and Helliker, 2010), which was attributed to competition between NH₄⁺-N and CH₄ by methanotrophic bacteria (Liu et al., 2017). However, it was still unclear that CH₄ uptake was affected in opposite or synergistic ways by N addition and low pH (i.e. much below the optima (pH 4.6-6.8)) (Benstead and King, 2001; Mosier et al., 1999). The overall N effect on CH₄ uptake varied (from CH₄ sink to source) substantially, which could be related to N enrichment and/or soil acidification.

Ecosystem respiration comprises heterotrophic respiration and autotrophic respiration. A recent study showed that soil pH played a greater role in controlling soil respiration than soil N availability in a temperate grassland (Chen et al., 2016). In

particular, heterotrophic respiration was decreased due to the inhibiting effect on microbial activity and abundance of decreasing pH (Chen et al., 2016). In addition, vegetation was also an important determinant of soil respiration because of the importance of root structure, in addition to the relative abundances of dominant soil bacterial taxa in an upland grassland soil (Thomson et al., 2010). Goulding et al. (1998) reported a much larger decrease in species richness in the long-term Park Grass Experiment receiving NH_4^+ -N than that receiving NO_3^- -N, which indicated that N availability and acidification both had a large impact on species composition. Therefore, N enrichment and soil acidification, through their impact on species composition, both have a major impact on ecosystem carbon balance.

The temperate semi-arid grassland of Inner Mongolia in northeast China covers an area of 3.1×10^8 ha, accounting for 13% of the global grassland and 78% of Chinese grassland (Wang et al., 2005). It has experienced significant anthropogenic N deposition over recent decades (Lu and Tian, 2007). To date, the prediction of GHG sink strength as affected by the N load has mostly relied on short-term experiments (Pinder et al., 2012; Chen et al., 2013). However, long-term experiments would more accurately characterized the response of the GHG sink to N deposition and N-induced reduced pH (Gomez-Casanovas et al., 2016). Long-term N addition and induced low pH are expected to have opposite or synergistic effects on N_2O and CH_4 emissions and R_e , but which made the greater contribution, and which other factors controlled emissions, is still not clear. Therefore, we established a long-term field experiment in 2005, applying N to a semi-arid grassland soil. This has resulted in a decrease of soil pH from 7.12 to between 5.49 - 4.64 after 11 years (Hao et al., 2018). These changes are expected to have had a profound impact on N_2O and CH_4 fluxes and ecosystem respiration. We have therefore measured these in this now 13-year old N addition experiment from May 2017 to October 2018 to: (i) clarify the responses of N_2O and CH_4 fluxes and ecosystem respiration to long-term increased N addition and N-induced low soil pH, and (ii) identify the main controlling factors of N_2O and CH_4 fluxes and ecosystem respiration caused by long-term increased N addition. We hypothesized the following: (1) N_2O emission is mainly regulated by soil N content rather than N-induced low pH; (2) N-induced low pH reduced soil CH_4 uptake and

ecosystem respiration.

Materials and methods

2.1. Study site

The experiment was established in a semiarid temperate steppe in Duolun County (116°17'E, 42°02'N, 1324 m above sea level), Inner Mongolia, China. The mean annual precipitation from 2006 to 2016 was 316 mm, and the 84% of annual rainfall occurring during the growing season (June to September). The annual average temperature was 3.3°C. The local N deposition was approximately 12 kg N ha⁻¹ yr⁻¹ (58% NH₄⁺, 42% NO₃⁻) during 2010 and 2014 according to Xu et al. (2015). The soil consists of 62.8% sand, 20.3% silt, and 16.9% clay and is classified as a Gelic Cambisol (Hao et al., 2018). The bulk density of the topsoil (0-10 cm) was 1.31 g cm⁻³ and the soil pH was 7.12 in 2005 (Song et al., 2012). The common grass-forb-legume classification was used to assign species (Hector et al., 1999; Tilman et al., 1997). The total N content, soil organic carbon (SOC) content and total P at 0-10 cm depth were 1.3 ± 0.03 g kg⁻¹, 12.3 ± 0.34 g kg⁻¹, 0.28 ± 0.06 g kg⁻¹, respectively (Wan et al., 2009).

2.2. Experimental treatments

A randomized block design was used with three N addition treatments. Each treatment comprised four blocks of 5×5 m with an 1 m wide buffer zone and four replicates. Nitrogen as ammonium nitrate was applied at rates of 0, 120 and 240 kg N ha⁻¹ yr⁻¹, identified as N₀, N₁₂₀, N₂₄₀. The ammonium nitrate was applied in 2 equal splits in mid-June and mid-July from 2005-2016. In 2017 and 2018, half of the N was applied on June 17th and half on July 18th. It was evenly sprayed to ensure uniform distribution. N₂O and CH₄ fluxes and ecosystem respiration were measured from May 2017 to October 2018.

2.3. Measurements

2.3. Measurement methods

N₂O and CH₄ fluxes and ecosystem respiration (R_e) were measured using static

chambers (diameter 25 cm, height 17 cm) made of stainless steel, inserted 10 cm into the soil. Fluxes were measured on all 12 plots, four replicates per treatment. Gas samples were collected from the headspace of each static chamber at 0, 10, 20 and 30 min after closing the chamber between 10:00 and 12:00 am (GMT+8) when fluxes were approximately the diurnal average (Yue et al., 2016). Fluxes were measured once or twice a week. Samples were analyzed using a gas chromatograph (GC; Agilent 7890A, Agilent Technologies, Santa Clara, CA, USA) equipped with a flame ionization detector for determining CH₄ and CO₂ (R_e), while N₂O was measured with an electron capture detector. Fluxes were calculated from the measured concentrations of the gases, based on a first order differential linear or non-linear equation, and were corrected for temperature and pressure (Liu et al., 2010). The non-linear calculation of fluxes corrected the suppression of emissions from gas accumulation inside the chambers (Zhang et al., 2014).

Soil samples were collected on May 13th, July 9th, July 16th, July 18th, July 19th, July 28th, Aug. 6th and Aug. 19th in 2017 from near the static chambers to a depth of 5 cm using a 3 cm diameter auger. Roots were collected from the 0-50 cm layer using a 7-cm soil corer on May 13th, July 14th and Aug. 19th in 2017. Dissolved organic carbon (DOC) was extracted from the soil samples with deionized water (soil:water ratio 1:10) after shaking on an orbital shaker at 5000 rpm for 5 min, and analyzed using a TOC analyzer (Multi N/C 3100, Jena, Germany)(Jones and Willett, 2006). Soil pH was measured in a 1:5 soil:deionized water suspension using a pH Meter (Seven Easy, Mettler-Toledo, Switzerland). Sub-samples were extracted in 0.01 mol/L CaCl₂ solution (soil:water ratio of 12:100) and shaken for 1 hour. The suspension was filtered and samples analyzed for NO₃⁻-N and NH₄⁺-N by continuous-flow auto-analyzer (Seal AA3, Germany). Soil water content and temperature at 5 cm depth were recorded using a TDR probe and 330 Soil Temperature Meter at the same time that gas samples were collected. The aboveground biomass (AGB) and species numbers of grasses, forbs and legumes were measured in late August 2017 in 1 m² quadrats. Water-filled pore space (WFPS, %) was calculated by the method of Yang et al. (2018).

2.4. Statistical analyses

Analysis of variance (ANOVA) with a least significant difference test (LSD, $P < 0.05$) was used to test for differences in pH, soil NO_3^- -N and NH_4^+ -N content, R_e , N_2O and CH_4 fluxes between the control and treatment plots. In addition, structural equation models (SEM) were used to search for relationships between N_2O , CH_4 , R_e and soil temperature/moisture, soil NO_3^- -N and NH_4^+ -N contents, pH, root and forb biomass. The effects of N deposition on R_e , N_2O and CH_4 fluxes were analyzed by one-way repeated measures ANOVA. All statistical analyses were conducted using SPSS (version 20.0) with statistical significant differences set at $P < 0.05$. SEM analyses were carried out using AMOS 22.0 (Amos Development Corporation, Chicago, IL, USA). All figures were drawn using Sigmaplot (version 12.5).

3. Results

3.1. Impact of long-term increased N addition on soil properties and plant composition

Soil temperature varied greatly during the growing season, reaching its maximum in July 2017 (26.8°C) (Fig. 1a). Water-filled pore space (WFPS, %) at 5 cm depth was increased by 0.32-1.77% by long-term N addition, but soil temperature was reduced by 1.35 - 1.72°C (Fig. 1a). Soil NH_4^+ -N was increased in the N_{120} and N_{240} treatments by 3 and 9 times, respectively, NO_3^- -N contents by 2 and 4 fold, respectively (Table 1), and pH was significantly reduced from 7.12 to 4.49 - 4.19 (Table 1). The underground biomass (UGB) and aboveground biomass (AGB) of plants were significantly increased by long-term N addition. DOC increased and species richness was reduced significantly in the N_{120} and N_{240} treatments (Tables 1 and 2).

3.2. N_2O emission

A small emission of N_2O from the control plots was measured, ranging from 0.59 to $16.32 \mu\text{g N m}^{-2} \text{h}^{-1}$ (average $3.58 \pm 0.48 \mu\text{g N m}^{-2} \text{h}^{-1}$) (Fig. 1b), being highest in July and lowest in October (Fig. 1b). In contrast, no significant emissions were observed in the non-growing season, the average emission rate was $2.55 \pm 0.29 \mu\text{g N m}^{-2} \text{h}^{-1}$ (Fig. 1b). N_2O emission was significantly increased by long-term N addition (Table 3), especially in the N_{240} treatment, where the maximum flux reached $88.51 \mu\text{g N m}^{-2} \text{h}^{-1}$ (Fig. 1b). A

significant cumulative effect of N addition on N₂O emission was found in the growing season (Table 3), which is consistent with the results of SEM, showing that available N was the most important controlling factor to N₂O emission, rather than soil pH (Fig. 2). Significant positive correlations of N₂O emission were observed with both soil temperature and soil moisture (Fig. 2).

3.3. CH₄ uptake

A rapid rate of CH₄ uptake in the N₀ plots was observed (13.0 - 82.3 µg C m⁻² h⁻¹) over the growing season, with an average uptake rate of 41.6 ± 2.4 µg C m⁻² h⁻¹ (Fig. 1c). There was a large seasonal variation, with a maximum uptake in August and a minimum in October (Fig. 1c). CH₄ uptake was significantly reduced by long-term increased N addition ($p < 0.0001$; Table 2): the average uptake in the N₁₂₀ and N₂₄₀ treatments was 30.8 ± 1.9 and 18.5 ± 1.8 µg C m⁻² h⁻¹ (Fig. 1c), respectively, and the fluxes decreased with the increasing rate of N addition. Inhibiting effects of soil moisture, NH₄⁺-N, NO₃⁻-N content, pH and plant roots were showed by SEM, among which soil pH made the greatest contribution (Fig. 3). A significant cumulative effect on CH₄ uptake was found from N addition in growing season (Table 2), but there were no significant correlations of CH₄ uptake with soil NH₄⁺-N content ($P > 0.05$, Fig. 3).

3.4 Ecosystem respiration (R_e)

Ecosystem respiration (R_e) in the control treatment was large, averaging 62.06 ± 7.25 mg C m⁻² h⁻¹ and ranging from 4.94 to 167.36 mg C m⁻² h⁻¹. It had a unimodal structure during the growing season, with a maximum in July and minimum in May (Fig. 1d). Average R_e of the N₁₂₀ and N₂₄₀ treatments were 61.01 ± 7.01 and 43.77 ± 4.67 mg C m⁻² h⁻¹, respectively. There was a significant reduction of the average R_e flux at N₂₄₀ ($p = 0.007$, Table 3) but not N₁₂₀ compared to N₀ (Fig. 1d). Structural Equation Modeling indicated that the aboveground biomass of forbs was the most important controlling factor of R_e, followed by soil temperature (Fig. 4). Soil NO₃⁻-N content and pH had indirect effects on R_e via their effect on forb biomass (Fig. 4).

4. Discussion

4.1. N_2O emission

The temperate grassland acted as a small source of N_2O , with the cumulative emission of N_2O from N_0 plots in the growing season being $0.034 \pm 0.004 \text{ kg N ha}^{-1}$ (Fig. 1b), smaller than that measured on nine other ungrazed steppe sites in Inner Mongolia ($0.06 \pm 0.03 \text{ kg N ha}^{-1}$) (Wolf et al., 2010). No significant N_2O emission was observed during the spring thaw (Fig. 1b), which may be attributed to there being less snow in the winter season than usually falls at the site, and so no anaerobic environment was created. This is different from Wolf et al (2010), who found that N_2O emission accounted for 72% of annual fluxes during the spring thaw in another temperate grassland, but consistent with Li et al. (2012), who reported only 6.6% of annual N_2O fluxes from the spring thaw in alpine grasslands of the Tianshan Mountains. Long-term N addition significantly increased N_2O emissions (Fig. 1b). SEM showed that nitrification was the main process producing N_2O (Fig. 2), supported by WFPS always being $< 15\%$ (Fig. 1a) and consistent with results showing that nitrification dominates N_2O emissions when WFPS is $< 70\%$ (Davidson, 1992; Zhu et al., 2013).

Typically, The low nitrification rate in acidic soils is due to shift in nitrifiers communities composition and limited by lack of the active nitrifier enzyme ammonia monooxygenase (AMO)(Norton and Stark, 2011). A laboratory experiment showed that their growth was stopped at a $\text{pH} < 5.5$ (De Boer and Kowalchuk, 2001). Our results indicate the dominance of $\text{NH}_4^+\text{-N}$ in controlling N_2O emissions rather than soil pH (Fig. 2). This could be because the reduced pH did not dramatically shift the N-inducing function and diversity of ammonia oxidizers (Ying et al., 2017), indicating the predominant role of N rather than low pH under long-term fertilization. In addition, soil temperature was also an important controlling factor of N_2O emissions, which is consistent with other results (Li et al., 2018), and because the activity of ammonia oxidizers is enhanced by soil temperature (Ying et al., 2017). Overall, N_2O emissions from these grassland soils were mainly controlled by soil $\text{NH}_4^+\text{-N}$, soil temperature and WFPS instead of pH.

4.2. CH₄ uptake

A large uptake rate of CH₄ was observed (13 - 82.3 $\mu\text{g C m}^{-2} \text{ h}^{-1}$) in the control plots over the growing season, with an average of $43.8 \pm 2.1 \mu\text{g C m}^{-2} \text{ h}^{-1}$ (Fig. 1c). Large temporal variation was observed, with the maximum uptake in August and minimum in January (Fig. 1c), which was consistent with other research in the same region (Chen et al., 2013; Liu et al., 2008). Temperate grassland is proved to be an atmospheric CH₄ important sink (Yu et al., 2017; Rong et al., 2015), CH₄ uptake was significantly reduced by long-term increased N addition, supporting our hypothesis, with uptake on the N₁₂₀ and N₂₄₀ plots being 33.4 ± 1.8 and $23.7 \pm 2.1 \mu\text{g C m}^{-2} \text{ h}^{-1}$, respectively. The N₂₄₀ treatment reduced uptake significantly by 46% ($P < 0.0001$; Table 2; Fig. 1). SEM showed soil NO₃⁻-N content to be the main controlling factor for CH₄ uptake (Fig. 3), because it increases soil redox potential and generates strong osmotic pressure effects (Treseder, 2008). Moreover, excessive NO₃⁻-N is toxic to *Methanotrophic* bacteria (Yue et al., 2016; Xu and Kazuyuki, 2007). In addition, long-term increased N addition ($>120 \text{ kg N ha}^{-1} \text{ yr}^{-1}$) has been found to cause a significant shift in bacterial community composition and a decrease in bacterial OTU richness in the surface soil (Zeng et al., 2016), which may change the composition of methane-oxidizing bacteria, reducing CH₄ uptake.

In contrast, no significant impact of soil NH₄⁺-N content on CH₄ uptake was observed (Fig. 3), which contradicts many previous studies (Aronson and Helliker, 2010). This was because the process of ammonia oxidation was inhibited by the decrease in soil pH, which weakens the ability of methanotrophic bacteria to oxidise CH₄ (Chen et al., 2013). The pH value (4.19 - 4.49) in our N-treated plots was much lower than the optimum pH value (between 4.6 and 6.8) for methanotrophic activity (Benstead and King, 2001). CH₄ uptake was also significantly reduced by increasing WFPS and root biomass (Fig. 3), which can be attributed to the inhibition of CH₄ diffusion by soil water content and root growth (Fig. 1a). Overall, long-term N addition reduced the CH₄ sink in this semi-arid grassland, but the CH₄ uptake rate was sensitive to changes in pH. Soil NO₃⁻-N and pH were the main controlling factors rather than NH₄⁺-N.

4.3 Ecosystem respiration (R_e)

R_e averaged $76.79 \pm 6.03 \text{ mg C m}^{-2} \text{ h}^{-1}$ in the N_0 treatment, ranging from 4.94 to 167.36 $\text{mg C m}^{-2} \text{ h}^{-1}$ and showing strong temporal variation with maximum values in July and minimum values in February (Fig. 1d). This is consistent with most research in Chinese grasslands, where soil temperature is the most important controlling factor (Chen et al., 2013; Dima et al., 2016). R_e was not significantly reduced by long-term N addition, which contradicts our hypothesis (Table 3). This is inconsistent with some short- or long-term N addition experiments due to N suppression to root and heterotrophic respiration (Huang et al., 2015; Zong et al., 2017; Leff et al., 2015). Dima and Chen (2016) found that soil acidification exerted a greater control than soil available N content on R_e in a long-term N enrichment experiment, which was confirmed by our results (Fig. 4). In addition, soil acidity and fertility affect root structure and fine root mortality through different species preferences for N (Buckeridge and Jefferies, 2007; Chen et al., 2015). Plant activity is an important driver of both components of heterotrophic and autotrophic respiration in prairie grassland ecosystem (Gomez-Casanovas et al., 2012). Carbon allocation to the roots of plants is decreased by long-term N addition, which also inhibits root growth and microbial respiration (Phillips and Fahey, 2007). In addition, plant species composition at our site has been significantly changed by long-term N addition (Hao et al., 2018). R_e correlated significantly and negatively with forb biomass (Fig. 4) but significantly positively with NO_3^- -N. In 2017, forb biomass accounted for 68% and 82% of total plant biomass in the N_{120} and N_{240} treatments, respectively, and contributed most in reducing R_e , because forbs with fine root diameter and small root mass, which can be ascribed to the salt tolerance (nitrate enrichment) (Smirnov and Stewart, 1985), generated less root respiration than grasses (Leuschner et al., 2004). Overall, greenhouse gas fluxes have been significantly changed by long-term increased N addition, which will produce a strong positive feedback to climate warming.

Conclusion

This study provides new insights into the impacts of long-term N addition and N-induced acidification on GHG emissions in a degraded temperate grassland. Long-term

N input degrades temperate grassland due to soil N enrichment and acidification, and simultaneously increases soil N₂O emissions, reduces CH₄ consumption, but has no significant impact on ecosystem respiration. NH₄⁺-N makes the dominant contribution to N₂O emission, rather than N-induced acidification; NO₃⁻-N and acidification together control CH₄ uptake, and ecosystem respiration is determined by forbs, but indirectly by NO₃⁻-N content and soil pH. These findings emphasize the roles and contributions of soil N availability and induced acidification to GHG emissions, especially in temperate grassland in northern China which has experienced elevated N deposition and serious acidification, and which will generate a strong negative feedback to climate change.

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Figure 1 Variation from April 2017 to October 2018 in soil moisture and soil temperature (a), fluxes in N_2O (b) and CH_4 (c), and ecosystem respiration (R_e) (d) (mean, $n=4$) in an Inner Mongolian grassland. The soil temperature and moisture in winter could not be measured because the soil was frozen.

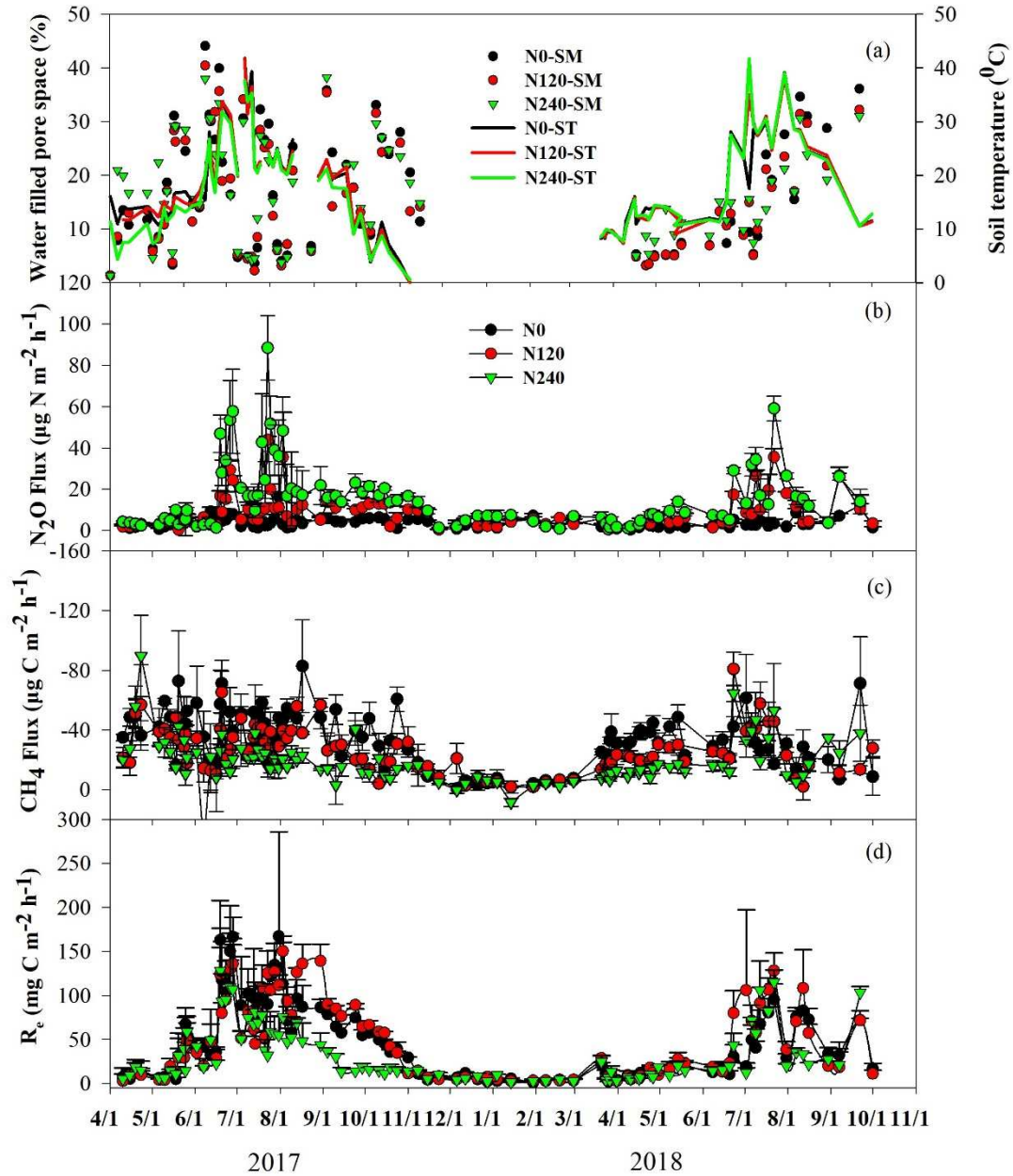
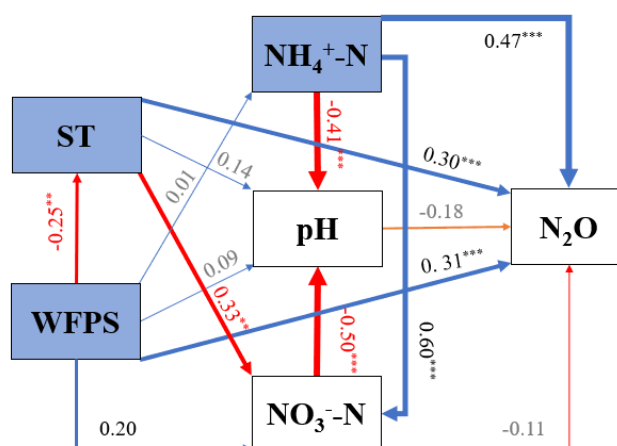
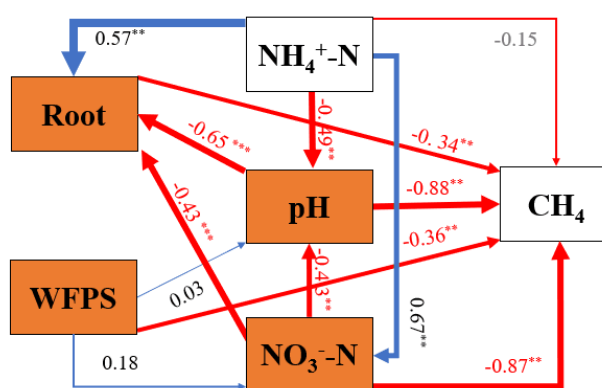


Figure 2 The effects on N_2O emissions ($n=28$) of soil moisture, temperature, pH, soil NO_3^- -N and NH_4^+ -N contents as tested by structural equation modeling (SEM). Single-headed arrows show that the impact of different significant controls on N_2O emission were analyzed. The blue arrows represent positive effects and red arrows negative effects. The width of the arrows indicates the strength of the relationship. The numbers are standardized path coefficients, which show the significance of the variables in the model. Goodness-of-fit statistics for the model are shown below the model. *, ** and *** indicate significant effect at $P < 0.05$, 0.01, and 0.001, respectively.



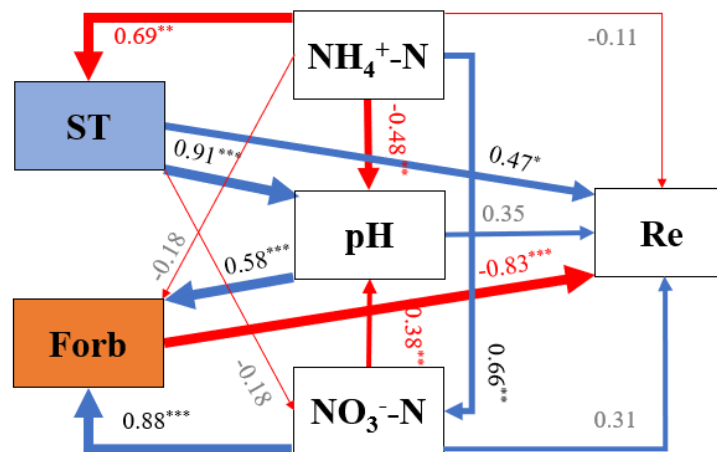
$\chi^2=6.028$, $p=0.064$, $df=1$, $\text{RSMEA}=0.017$, $\text{AIC}=58.082$

Figure 3 The effects on CH₄ uptake (n=28) of soil moisture, root biomass, pH, soil NO₃⁻-N and NH₄⁺-N contents as tested by structural equation modeling (SEM). Single-headed arrows show that the impact of different significant controls on CH₄ uptake were analyzed. The blue arrows represent positive effects and red arrows negative effects. The width of the arrows indicates the strength of the relationship. The numbers are standardized path coefficients, which show the significance of the variables in the model. Goodness-of-fit statistics for the model are shown below the model. *, ** and *** indicate significant effect at $P < 0.05$, 0.01, and 0.001, respectively.



$X^2=0.619$, $p=0.734$, $df=2$, $RSMEA=0.017$, $AIC=65.152$

Figure 4 The effects on ecosystem respiration (R_e) (n=28) of soil temperature, forb biomass, pH, soil NO_3^- -N and NH_4^+ -N contents as tested by structural equation modeling (SEM). Single-headed arrows show that the impact of different significant controls on R_e were analyzed. The blue arrows represent positive effects and red arrows negative effects. The width of the arrows indicates the strength of the relationship. The numbers are standardized path coefficients, which show the significance of the variables in the model. Goodness-of-fit statistics for the model are shown below the model. *, ** and *** indicate significant effect at $P < 0.05$, 0.01, and 0.001, respectively.



$X^2=17.249$, $p=0.07$, $df=6$, $RSMEA=0.048$, $AIC=77.249$

Table 1 NO_3^- -N, NH_4^+ -N, soil pH and dissolved organic carbon (DOC) in the topsoil (0-5 cm) of the different treatments at the sampling dates in 2017.

		13/5	15/6	9/7	16/7	18/7	28/7	6/8	29/8
NH_4^+ -N	N_0	4.87±0.65a	6.67±0.87a	4.21±0.37a	7.39±1.23a	7.39±1.23a	6.74±3.89a	7.86±0.89a	5.30±2.82a
	N_{120}	4.69±0.003a	5.54±0.67a	23.57±5.39b	24.95±4.26ab	23.65±10.80b	39.53±8.99b	28.51±3.28b	49.21±19.67b
	N_{240}	8.19±0.24b	6.78±1.83a	60.57±20.06c	118.90±12.08c	83.05±25.87c	86.47±4.27c	98.76±21.49c	100.54±6.59c
NO_3^- -N	N_0	2.99±0.77a	17.65±0.89a	28.78±7.06a	6.50±0.54a	6.50±0.54a	13.78±3.36a	3.99±1.41a	11.65±5.28a
	N_{120}	5.73±3.47a	20.64±5.49a	50.24±10.05b	23.48±1.24b	33.86±16.19b	20.08±4.63ab	28.31±4.88b	17.95±1.50a
	N_{240}	10.02±0.43b	26.56±7.38a	43.71±6.76b	54.11±20.00b	81.96±20.14c	28.31±3.50b	47.09±9.37c	40.99±4.10b
pH	N_0	6.81±0.14c	6.89±0.16b	6.65±0.37c	6.86±0.22b	6.59±0.06c	6.35±0.07a	6.17±0.04c	6.32±0.09c
	N_{120}	5.49±0.07b	6.66±0.36b	5.06±0.06b	5.22±0.06a	4.88±0.04b	5.01±0.08a	4.82±0.18b	4.37±0.14b
	N_{240}	4.64±0.02a	4.73±0.06a	4.83±0.03a	4.62±0.06a	4.33±0.02a	4.68±0.06a	4.47±0.13a	4.17±0.06a
DOC	N_0	41.49±10.72a	48.65±8.67a	69.85±6.19a	97.29±16.98a	82.68±14.84a	133.79±6.58a	82.36±10.98a	68.46±4.01a
	N_{120}	50.99±3.78a	59.74±4.45a	91.04±9.98b	124.88±6.87b	121.19±20.96b	114.77±16.88a	97.41±14.97a	87.37±4.17b
	N_{240}	60.06±5.64b	69.34±9.43c	100.51±12.78c	115.59±3.79ab	140.83±40.41b	107.73±13.51a	152.53±19.60ab	87.35±5.29b

Note: Values in the same column without same letters indicate significant differences ($P < 0.05$)

Table 2 Annual mean emissions of N₂O, CH₄ and CO₂ (R_e), forb biomass, aboveground biomass (AGB) and the increase of underground biomass (UGB) (mean ±SE, n=4) in August 2017 on the three treatments during the observation period. Different letters in the same column indicate significant differences ($P < 0.05$).

Treatments	N ₂ O (kg N ha ⁻¹)	CH ₄ (kg C ha ⁻¹)	CO ₂ (kg C ha ⁻¹)	Forb biomass (g m ⁻²)	AGB (g m ⁻²)	UGB (g m ⁻²)	Species richness
N ₀	0.36 ± 0.05a	-3.05 ± 0.14a	3090.84 ± 158.21b	101.74±9.20a	135.97±9.65a	334.07±29.90a	18.80±0.93a
N ₁₂₀	0.59 ± 0.07a	-2.32 ± 0.10b	2877.68± 254.71ab	111.87±5.03a	187.35±16.31b	897.44±91.89b	10.00±1.25b
N ₂₄₀	1.15 ± 0.11b	-1.46 ± 0.17c	2408.15 ± 211.44a	198.92±21.76b	220.67±26.72b	1138.15±386.41b	6.20±0.47c

Table 3 Repeated measures ANOVA analysis of variance with F and P values of the effects of long-term nitrogen (N) addition on N₂O emission, CH₄ uptake and ecosystem respiration (R_e); n indicates sample size.

Source of variation	N ₂ O			CH ₄			R _e		
	df	F	P	df	F	P	df	F	P
Date	3.276	19.432	<0.001**	4.202	8.849	<0.001**	2.841	21.609	<0.001**
n	2	100.386	<0.001**	2	12.158	0.008**	2	3.712	0.089
Date*n	6.553	4.786	0.003**	8.404	2.339	0.048*	5.682	1.817	0.158

Highlights

- We studied the impacts of long-term N addition on a temperate grassland
- CH₄ uptake was significantly reduced by long-term N addition
- N₂O emission was controlled mainly by soil ammonium N rather than soil pH
- CH₄ uptake was affected mainly by soil pH and nitrate N content
- Ecosystem respiration was impacted mainly by production of forbs.